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For: NOVEL SEQUENCES AND THEIR USE

CLAIM TO PRIORITY

Assistant Commissioner for Patents
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Date: January 23, 2002

Sir:

Applicant(s) herewith claim(s) the benefit of the priority filing date of the following application(s) for the above-entitled U.S. application under the provisions of 35 U.S.C. § 119 and 37 C.F.R. § 1.55:

Country	Application No.	Filed
SWEDEN	0002189-9	June 9, 2000

Certified copy(ies) of the above-noted application(s) is(are) attached hereto.

Respectfully submitted,

YOUNG & THOMPSON

Benoit Castel

Benoit Castel, Reg. No. 35,041

745 South 23rd Street
Arlington, VA 22202
Telephone (703) 521-2297

BC/mdp

Attachment(s): 1 Certified Copy(ies)

PRV

PATENT- OCH REGISTRERINGSVERKET
Patentavdelningen

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For: NOVEL SEQUENCES AND THEIR USE
Our Ref.: 45069
1 of 1

Intyg
Certificate

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This is to certify that the annexed is a true copy of the documents as originally filed with the Patent- and Registration Office in connection with the following patent application.

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Christina Vängborg

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PATENT- OCH REGISTRERINGSVERKET
SWEDEN

Postadress/Adress
Box 5055
S-102 42 STOCKHOLM

Telefon/Phone
+46 8 782 25 00
Vx 08-782 25 00

Telex
17978
PATOREG S

Telefax
+46 8 666 02 86
08-666 02 86

New method and assay

The present invention concerns the field of metabolic diseases, in particular diabetes, and the research efforts around these diseases. The present invention makes available a novel method and assay for screening drug candidates and for differentiating between and evaluating insulin sensitizing substances.

Background of the invention

Diabetes mellitus is a complex disorder of carbohydrate, fat, and protein metabolism that is primarily a result of a relative or complete lack of insulin secretion by the beta cells of the pancreas or of defects of the insulin receptors. The various forms of diabetes are divided in two categories, the most frequent being juvenile-onset diabetes or Type I insulin-dependent diabetes mellitus (IDDM) and adult-onset diabetes or Type II non-insulin-dependent diabetes mellitus (NIDDM). Both diseases, even when correctly diagnosed and medicated, require life-long medication, good patient compliance, a careful diet and frequent medical observation to avoid potentially serious sequelae.

Thiazolidinediones (TZD) are a recently identified class of antidiabetic agents which act by improving insulin sensitivity in both different animal models of obesity and diabetes (1-3) as well as in man (4, 5). In addition to improving the glucose and insulin levels, the circulating free fatty acids (FFA) and triglycerides are also lowered.

TZD promote fat cell differentiation and activate several adipocyte-specific genes such as the fatty acid binding protein, aP2, as well as the lipoprotein lipase. There is much recent evidence that TZD induce their diverse effects by binding to and activating the peroxisome proliferator activated receptor (PPAR)γ (reviewed in ref. 7). PPARγ is mainly expressed in the adipose tissue and exists as two isoforms, PPARγ1 and γ2. PPARγ1 is the major isoform and accounts for around 85 % of that in the adipose tissue. The isoforms differ in their NH_2-terminal end, with PPARγ2 having an additional 30 amino acids, and are generated from the same gene by mRNA splicing.

It is currently unclear how TZD improve insulin sensitivity since known PPARγ-regulated genes mainly involve adipocyte differentiation, lipid storage and metabolism (7).

Current hypotheses of the mechanisms for the direct insulin sensitizing effect of TZD include the formation of new, small and insulin-sensitive fat cells; the inhibition of TNFα production and, hence, its negative effects on insulin signaling or; as found in some experiments, increased GLUT4 expression in adipocytes although this requires activation of C/EBPα, as well. However, no clear and reproducible link to the intracellular signaling molecules for insulin has been found so far.

Prior art

Recently, Ribon *et al.* (13) reported a potential link in that TZD were found to increase the gene and protein expression of CAP, a c-Cbl associated protein which may be involved in the regulation of the tyrosine phosphorylation of c-Cbl by the insulin receptor. According to Mastick and Saltiel (14) c-Cbl, in turn, can interact with the kinase fyn to initiate phosphorylation of caveolin and other sequestered proteins (14).

There remains a need for methods for differentiating between insulin sensitizing substances and in particular specific markers for IRS-2 transcription. A problem to be solved is therefor inter alia how to screen the class of TZD compounds in respect of insulin sensitizing properties in different cells.

Another problem is how to improve the treatment of diabetes and in particular to develop new pharmaceuticals for this purpose.

Summary of the invention

The present inventor has examined in differentiated 3T3-L1 adipocytes the effect of PPARα and γ ligands on the expression of several key molecules for insulin signaling and action; IRS-1, IRS-2, PKB/Akt and GLUT4. It was surprisingly and unexpectedly found, that the only gene, which was rapidly and reproducibly increased was IRS-2, and this was also associated with an increased protein expression. Pioglitazone was found to induce transcription of the IRS-2 gene, resulting in a major transcript having a molecular weight of 7.2 kb. A minor transcript having a molecular weight of 8.2 kb was also frequently detected.

The above problems and shortcomings of the prior art are solved by invention as set forth in the attached claims, which are hereby incorporated in their entirety. Further advantages, embodiments and characteristics of the invention will be evident from the following description and examples.

Short description of the figures

The present invention will be disclosed in detail below, in the description and attached examples and figures, in which

Fig. 1 (A) top – shows the IRS-2 mRNA expression (Northern blots) in differentiated 3T3-L1 adipocytes cultured for 48 hrs with no additions (bas), 100 nM insulin (ins) or 10 μM pioglitazone (pio) in combination with 100 nM insulin. Actin mRNA is also shown for the same cells. **bottom** – shows IRS-2 mRNA levels from cells cultured for 48 hrs with no additions (bas), with 10μM pioglitazone (pio) with or without 100 nM insulin (pio + ins). **(B) top** – shows individual data from 5 experiments where IRS-2 mRNA was related to β-actin gene expression in the same cells (arbitrary units). Bars represent mean values. **bottom** - IRS-2 protein expression in differentiated 3T3-L1 cells cultured for 48 hrs with no additions (bas), with 100 nM insulin (ins) or with 10 μM pioglitazone (pio). The scanned data are also shown below (arbitrary units). **(C)** Time-course for IRS-2 mRNA expression in differentiated 3T3-L1 adipocytes cultured with 10 μM pioglitazone for the indicated times. The values represent % increase over non-stimulated control cells and are the means of two experiments.

Fig. 2 top – shows IRS-2 mRNA in differentiated 3T3-L1 adipocytes cultured for 48 hrs with 100 nM insulin (ins) alone or with 10 μM darglitazone (dar). **middle** – shows IRS-2 mRNA in cells cultured with 100 nM insulin (ins),10 μM of the PPARα agonist WY14643 (WY), with or without 100 nM insulin, and 10 μM pioglitazone (pio). **bottom** – shows IRS-2 mRNA from differentiated cells that had been cultured for 48 hrs with no additions (bas), 100 nM progesterone (prog) with or without 10 μM pioglitazone (pio) or 200 μM 8-BrcAMP with or without pioglitazone.

Fig. 3 (A) shows IRS-1 and PKB/Akt mRNA in differentiated 3T3-L1 adipocytes cultured for 48 hrs with no additions (bas), 100 nM insulin (ins) or 10 μM pioglitazone (pio) with insulin. **(B) top** - Individual data from 5 experiments (same as shown in Fig.1B) where IRS-1 mRNA

was related to β-actin gene expression in the same cells (arbitrary units). Bars represent mean values. **bottom** - IRS-1 protein expression in differentiated 3T3-L1 cells cultured for 48 hrs with no addition (bas), 100 nM insulin, 10 μM pioglitazone (pio) with or without 100 nM insulin. The scanned data are also shown below (arbitrary units).

Fig. 4 shows an immunoblot of serine- or threonine-phosphorylated PKB. Lysates were prepared from cells that had been cultured for 48 hrs as in Fig.3A, serum-starved for 3 hrs, and then stimulated with 100 nM insulin for 10 min. Following separation on 7.5 % SDS-PAGE, the proteins were immunoblotted with antibodies recognizing insulin-stimulated phosphoserine (PS-PKB) and phosphothreonine (PThr-PKB) PKB/Akt.

Description

In the present study, the present inventor demonstrates for the first time that PPARγ activation, but not PPARα, rapidly turns on the gene of the key signaling molecule IRS-2. This effect was initiated after 4 hrs, peaked after 24 hrs and remained elevated throughout the 48 hrs study. Furthermore, this effect appeared specific since, under the same conditions and in the same cells, no effects on IRS-1, PKB/Akt or GLUT4 were seen. Thus, it was not related to a general effect on cell differentiation by the PPARγ ligands. In addition, IRS-2 protein expression was also increased (~40 %) after 48 hrs and its signaling through the PI3-kinase and PKB/Akt pathway also tended to be increased. Since the IRS-2 gene activation was rapid and seen in the presence of the protein synthesis inhibitor, cycloheximide, the data suggest a direct effect of PPARγ activation on the IRS-2 promoter. However, this has to be directly tested in appropriate reporter assays.

The partial human IRS-2 gene and promoter were recently cloned and sequenced (19) but the complete murine promoter has not been reported. Interestingly, progesterone and cAMP were found to increase the expression of the IRS-2 gene in HeLa cells but GRE/PRE were not identified in the promoter. Sequencing the promoter identified multiple binding sites for several transcription factors such as Sp1, AP2 and CCAAT-box binding factor (19). However, the identified sequence in the human IRS-2 promoter (20) does not contain the typical AGGTCA binding sites for PPAR. Studies with appropriate reporter systems are necessary to clarify if PPARγ ligands directly activate the mouse and/or human IRS-2 genes. Interestingly,

preliminary studies by the present inventor have shown that pioglitazone also increases IRS-2 gene expression in human fat cells from type 2 diabetic individuals (unpublished observations).

Insulin alone did not change the IRS-2 gene expression and there was no synergistic effect between TZD and insulin. These latter data must be interpreted with some caution since the serum in the culture medium contains insulin. Surprisingly, no effect of either progesterone or cAMP on IRS-2 gene expression in 3T3-L1 cells could be seen. This is in contrast to recent data in HeLa cells where these agents increased IRS-2 mRNA levels (18) but this may be an indirect effect (19) and not seen in all cells.

Similar to IRS-2, chronic exposure to insulin did not change IRS-1 gene expression. However, in contrast to IRS-2, IRS-1 protein expression was reduced after chronic insulin stimulation in the 3T3-L1 cells as also reported by others (21-22). This effect of chronic marked hyperinsulinemia is then probably due to an increased protein degradation. Recent studies have shown that chronic exposure to insulin leads to an increased serine/threonine phosphorylation through the PI3-kinase and PKB/Akt pathway (21-23). IRS-1 is then degraded through the proteasomal pathway probably as a result of ubiquination (22).

Cells chronically exposed to the high insulin concentration also had an impaired acute response to insulin, which probably was due to an impaired activation of PI3-kinase and the down-stream signaling (22, 23). This was seen by the reduced phosphorylation of PKB on both serine and threonine sites. The addition of pioglitazone did not clearly improve the insulin effect. These data are in agreement with the concept that IRS-1 is the major docking protein for PI3-kinase in response to insulin in both 3T3-L1 cells (24) as well as in human fat cells (16) and that IRS-2 functions as a true "back-up" protein.

However, it is still surprising that the increased IRS-2 protein expression was unable to clearly improve the PI3-kinase activity and down-stream signaling. These results are similar to recent findings in IRS-1 "knock-out" cells (25) as well previous findings by the present inventor in human fat cells from Type 2 diabetic subjects, where IRS-2 expression is normal but IRS-1 protein expression is reduced ~70 % (16). PKB activation and phosphorylation remained impaired in these cells even in the presence of supramaximal insulin concentrations (25, 26). Whether longer exposure to TZD than the 48 hrs used in this study will further increase IRS-2 protein expression and restitute the insulin response remains to be established.

Furthermore, it will be of interest to see if the low IRS-1 expression in fat cells from Type 2 diabetic subjects (16) as well as in the cohort the present inventor have identified of non-diabetic but markedly insulin-resistant individuals with a genetic predisposition for diabetes (27) will be compensated for by an increased IRS-2 expression following TZD.

In summary, the present data show for the first time a clear link between PPARγ ligands and the insulin signaling cascade in that TZD rapidly increase IRS-2 gene (and protein) expression in 3T3-L1 cells. Since low IRS-2 appears to play a profound role in the development of diabetes (28) these data suggest that the antidiabetic effect of TZD may be mediated through this effect. It will also be of great interest to see if TZD can influence β-cell growth and/or apoptosis since a stunning effect of IRS-2 gene disruption is seen on pancreatic β-cell development (28). Thus, although IRS-2 can also be used by insulin as a docking protein for PI3-kinase activation, it may play an even more profound role in the signaling and effect of cytokines and growth factors.

The expression of several genes involved in insulin signaling and action were examined after 4-48 hrs exposure to different concentrations of pioglitazone (pio). However, only the IRS-2 gene expression was consistently increased. The IRS-2 mRNA included a major ~7.2 Kb band but a minor band at ~8.2 Kb was also frequently seen.

It is of commercial value to be able to control the IRS-2 level to thereby regulate insulin sensitivity in adipocytes and other cells to normalize metabolism in type II diabetics. The findings disclosed above constitute a basis for finding such regulating substances, not only for human adipocytes but also for different, specific human cells such as hepatic cells, pancreatic cells or muscle tissue cells.

According to one embodiment of the invention, IRS-2 transcripts are used as markers to screen the class of TZD compounds in respect of insulin sensitizing properties in different cells. The detection of the transcripts is facilitated by coupling at least one IRS-2 transcript to a suitable reporter, e.g. a fluorescent reporter molecule.

According to another embodiment, among the IRS-2 transcripts, at least one of the above 7.2 and 8.2 kb transcripts is used as a specific marker to screen and find compounds possessing insulin sensitizing properties in different cells. The detection of said, at least one transcript, is facilitated by coupling said at least one IRS-2 transcript to a suitable reporter, e.g. a fluorescent reporter molecule.

According to yet another embodiment, at least one of the above 7.2 and 8.2 kb transcripts is used as a specific marker to screen and find adipocyte specific insulin sensitizers using 3T3-L1 cells and subsequent detection of the at least one transcript. This detection is facilitated by coupling the IRS-2 transcript to a suitable reporter, e.g. a fluorescent reporter molecule. Suitable screening systems include, but are not limited to Northern blots, RT-PCR using specific primers and probes for IRS-2, solution hybridisation and RNA'ase protection assays.

Large scale screening, e.g. so called high throughput screening of chemical libraries can be performed with a reporter system, adapted for specific sequences of the IRS-2 promotor. Useful reporter sequences are luciferase or similar, sensitive assays.

According to a further embodiment of the invention, this at least one marker is used in a screening system to find and discriminate between insulin sensitizers acting on different cells, such as hepatic cells, muscle tissue cells and adipocytes.

The detection of the above at least one IRS-2 transcript is performed either qualitatively or quantitatively. Qualitative detection methods comprise any method where the presence or absence of a marker is determined, e.g. based on radiation, fluorescence, etc. Quantitative methods comprise any method where the amount of marker is determined, e.g. qualitative PCR or RT-PCR.

Preferably, the at least one IRS-2 transcript or information derived therefrom is/are used for the production of an assay for the screening of drug candidates in respect of their insulin sensitizing properties. Most preferably, said assay is constructed as an assay suitable for high throughput (HTP) screening, for example an assay adapted for the commonly used 96-well format, the 384-well format or denser formats, such as micro arrays or chips, carrying immobilised reagents on their surface.

Further, the present invention comprises a method, wherein the regulating elements regulating and/or contained in IRS-2 transcripts, in particular in the 7.2 and 8.2 kb transcripts, are used as drug targets to prevent or treat diabetes.

According to one embodiment of the invention, an IRS-2 transcript is used for the manufacture of a medicament. In particular, an IRS-2 transcript having a molecular weight of 7.2 kb or 8.2 kb is used for the manufacture of a medicament.

According to one embodiment of the invention, an IRS-2 transcript is used for the manufacture of a medicament for the treatment of diabetes. In particular, an IRS-2 transcript having a molecular weight of 7.2 kb or 8.2 kb is used for the manufacture of a medicament for the treatment of diabetes.

Further, the sequence information derived from an IRS-2 transcript can be used for the manufacture of a medicament, in particular a medicament for the treatment of diabetes.

Further, the sequence information derived from an IRS-2 transcript can be used in an assay for diagnosing diabetes and/or differentiating between various types or stages of the disease.

The results also support the conclusion, that thiazolidinediones, such as pioglitazone, have new specific modes of action and that they thus can as medicaments with specific therapeutic actions. The present invention thus comprises the use of thiazolidinediones, such as pioglitazone, for the manufacture of a medicament for regulating the insulin sensitivity of cells, e.g. for increasing the insulin sensitivity of adipocytes.

Examples

1. Materials and methods

1. 1 Cell Cultures

3T3-L1 fibroblasts were grown and differentiated into adipocytes according to Rubin *et al.* (15). At least 90 % of the cells had acquired the adipocyte phenotype 6 days after initiating differentiation. Eight days after differentiation, medium was changed and the various agents added for the times indicated in the Results.

To study the acute effect of insulin, the cells were serum – deprived for 3 hrs before adding 100 nM insulin for 15 min. Cell lysates were made using procedures previously described by Rondinone, et al. (16).

1. 2 Analyses of RNA

Total cellular RNA was isolated from cells with guanidinium thiocyanate, as described (17). Northern blot analyses were performed on total cellular RNA (30 µg) with labeled cDNA

probes made against β-actin as housekeeping gene, mouse IRS-1 (bp 1333-2335) and mouse IRS-2 (bp 2987-3325) (kindly provided by Drs. J. Pierce and L-M Wang, NCI, NIH), rodent GLUT4 (bp 121-2128, Accession Nr NM 001042, kindly provided by Dr. Sam W. Cushman, NIDDK, NIH) and PKB/Akt using a PCR fragment against PKBβ (bp 282-1130, Accession Nr M95936) in a common sequence for PKBα, PKBβ and PKBγ. 5' sequence CGAGAGGCCGCGACCCAACAC and 3' sequence AGGCGGCCGCACATCATCTCGTA were used as PCR primers.

1. 3 Immunoprecipitations and Immunoblotting

Cell lysates were prepared as described by Rondinone et al. 1997 (*16*). Equal amounts of protein were separated by SDS/PAGE, transferred and immunoblotted with appropriate antibodies against the specific proteins. Phosphotyrosines were immunoblotted with antibody PY99 (Transduction Laboratories, Lexington, NY), GLUT4 with an antibody kindly provided by Dr. Sam W. Cushman (NIDDK, NIH), IRS-1 and IRS-2 with antibodies from Upstate Biotechnology, Inc. (Lake Placid, NY) and PKB/Akt with antibodies from Biolab (Boston, MA).

Immunoprecipitations were performed as described (16) and individual proteins were detected by blotting with horseradish peroxidase – linked secondary antibodies and using enhanced chemiluminescence (Nycomed Amersham plc., UK).

2. Results

2. 1 Effects of PPAR agonists and/or insulin on IRS-1/2, PKB and GLUT4 gene and protein expression

The expression of several genes involved in insulin signaling and action were examined after 4-48 hrs exposure to different concentrations of pioglitazone (pio). However, only the IRS-2 gene expression was consistently increased. The IRS-2 mRNA included a major ~7.2 Kb band but a minor band at ~8.2 Kb was also frequently seen. It is not clear whether this represents alternative splicing of the same gene.

Fig. 1A (top) shows that insulin alone did not change IRS-2 gene expression while the addition of pioglitazone increased IRS-2 mRNA ~ 4-fold. This was due to an effect of pioglitazone alone and no further increase was seen by the addition of insulin (Fig. 1A - bottom). After 48 hrs, the IRS-2 mRNA levels were consistently increased 3-5-fold relative to β-actin mRNA (Fig.1B - top). IRS-2 protein expression was also increased by pioglitazone but not changed by insulin (Fig.1B - bottom). The average increase seen after 48 hrs with pioglitazone was 36 % (n=3).

Time-course experiments showed that the gene expression was already increased after 4 hrs, peaked after 24 hrs but remained elevated throughout the 48 hrs observation time (Fig. 1C). This increase was specific for IRS-2 and remained irrespective of whether the IRS-2 expression was related to β-actin, IRS-1 or PKB mRNA levels (data not shown). The present inventor also examined if the rapid effect of pioglitazone was direct or required protein synthesis by adding cycloheximide (40 μM) to the incubation medium for 8 and 24 hrs. However, pioglitazone also increased the IRS-2 mRNA expression in the presence of cycloheximide, suggesting a direct effect of pioglitazone on the IRS-2 gene (not shown).

The present inventor also examined whether a lower pioglitazone concentration increased IRS-2 mRNA expression. A similar effect was seen with 1 μM pioglitazone (average increase 230 %, n=2) as with 10 μM in the same experiments (average increase 292 %, n=2). Furthermore, darglitazone, another PPARγ ligand, induced a similar increase as pioglitazone (Fig. 2 - top) while a specific PPARα ligand (WY14643) was completely without effect (average increase 9 %, n=2) even at a high concentration (10 μM) and when insulin was added (Fig. 2 - middle).

Thus, these data show that PPARγ, but not PPARα, ligands increase IRS-2 gene expression in 3T3-L1 adipocytes. The inventor also tested whether progesterone or 8-BrcAMP, which have been shown to increase IRS-2 gene expression in HeLa cells (18), also altered the expression in 3T3-L1 cells. However, no effects were seen even with high concentrations of these agents. However, when combined, the stimulating effect of pioglitazone was again shown (Fig. 2 - bottom).

Neither insulin alone nor when combined with the PPARγ ligands, pioglitazone or darglitazone (not shown), altered the gene expression of IRS-1 or of PKB/Akt (Fig. 3A) in the same experiments. Similarly, no effect was seen with the PPARα agonist (data not shown).

The PCR fragment used for PKB hybridization gave two major bands (3.2 and 2.8 Kb) probably reflecting both the PKBα/Akt 1 and the PKBβ/Akt 2 genes. Individual results of IRS-1 mRNA expression from five experiments are shown in Fig. 3B.

Similar to IRS-1, there was no consistent increase in GLUT4 mRNA expression by pioglitazone after 48 hrs incubation (data not shown).

2. 2 Effect of TZD and/or insulin on IRS-1 protein expression and PKB/Akt phosphorylation

Fig. 3B (bottom) shows the chronic effects of pioglitazone and/or insulin on IRS-1 protein expression after 48 hrs. Pioglitazone alone did not change IRS-1 protein expression. However, in contrast to IRS-2 (Fig. 1B - bottom), IRS-1 protein expression was reduced by chronic stimulation with the high insulin concentration and this was not altered by the presence of pioglitazone (Fig. 3B - bottom).

In three experiments, insulin decreased IRS-1 protein expression by 33 % (range 25-39 %); this decrease remained unchanged when insulin and pioglitazone were combined (-31 %, range 18-39 %) while pioglitazone alone was without effect (+7 %). These differences in protein expression were also reflected by the amount of p85 co-immunoprecipitated with IRS-1 (not shown).

The present inventor also examined the acute effect of insulin on down-stream activation of PKB/Akt in cells that had been cultured with pioglitazone for 48 hrs, washed and serum-starved for 3 hrs in fresh medium. Similar to the gene (Fig. 3A), PKB/Akt protein expression was not changed by the presence of either pioglitazone and/or insulin for 48 hrs (not shown). Insulin (100 nM) was then added for 10 min to the serum-starved cells. As shown in fig. 4, the acute effect of insulin on PKB/Akt phosphorylation tended to be increased in cells cultured with pioglitazone alone.

In contrast, chronic exposure to insulin alone markedly reduced the acute effect of insulin on PKB/Akt phosphorylation. The addition of pioglitazone for 48 hrs did not improve the reduced insulin-stimulated phosphorylation of PKB/Akt induced by the chronic hyperinsulinemia but an improvement was seen after 96 hrs.

Further, preliminary studies performed by the present inventor, indicate that similar IRS-2 transcription is achieved in rat muscle cells L6. Likewise, experiments with human adipocytes indicate, that the results obtained with 3T3-L1 adipocytes are transferable to human cells. The present inventor has shown that TZD increase IRS-2 expression in human adipocytes after 24 hrs in culture. Thus, the results available at the priority date support a broad application of the invention.

Although the invention has been described with regard to its preferred embodiments, which constitute the best mode presently known to the inventor, it should be understood that various changes and modifications as would be obvious to one having the ordinary skill in this art may be made without departing from the scope of the invention as set forth in the claims appended hereto.

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Claims

1. An IRS-2 transcript, characterized in that it is induced by the addition of a thiazolidinedione, such as pioglitazone, to living cells.

2. An IRS-2 transcript having a molecular weight of 7.2 kb induced in differentiated 3T3-L1 adipocytes by the addition of a thiazolidinedione, such as pioglitazone.

3. An IRS-2 transcript having a molecular weight of 8.2 kb induced in differentiated 3T3-L1 adipocytes by the addition of a thiazolidinedione, such as pioglitazone.

4. Method for evaluating substances for insulin sensitizing properties *in vitro*, characterized in that a transcript according to claim 1 is used as a marker for insulin sensitizing action.

5. Method for evaluating substances for insulin sensitizing properties *in vitro*, characterized in that a transcript according to claim 2 is used as a marker for insulin sensitizing action.

6. Method for evaluating substances for insulin sensitizing properties *in vitro*, characterized in that a transcript according to claim 3 is used as a marker for insulin sensitizing action

7. Method according to any one of claims 4 to 6, characterized in that adipocytes are used as model cells.

8. Method according to any one of claims 4 to 6, characterized in that hepatic cells are used as model cells.

9. Method according to any one of claims 4 to 6, characterized in that muscle tissue cells are used as model cells.

10. Method according to any one of claims 4 to 6, characterized in that pancreatic cells are used as model cells.

11. Use of a transcript according to claim 1, or information derived therefrom, for the manufacture of a medicament.

12. Use of the transcript according to claim 1, or information derived therefrom, for the manufacture of a medicament for the treatment of diabetes.

13. Use of the transcript according to claim 2, or information derived therefrom, for the manufacture of a medicament.

14. Use of the transcript according to claim 2, or information derived therefrom, for the manufacture of a medicament for the treatment of diabetes.

15. Use of the transcript according to claim 3, or information derived therefrom, for the manufacture of a medicament.

16. Use of the transcript according to claim 3, or information derived therefrom, for the manufacture of a medicament for the treatment of diabetes.

17. An assay for the screening of substances in respect of their insulin sensitizing properties, characterized in that an IRS-2 transcript is used as marker for insulin sensitizing properties.

18. An assay for diagnosing diabetes and/or differentiating between various types or stages of the disease, characterized in that an IRS-2 transcript or sequence information derived therefrom is used in said assay.

19. Use of pioglitazone for the manufacture of a medicament for increasing the insulin sensitivity of adipocytes.

Abstract

Thiazolidinediones (TZD) improve insulin sensitivity in man as well as in different animal models of insulin resistance and Type 2 diabetes. However, no clear link to the insulin signaling events has been identified.

Using differentiated 3T3-L1 adipocytes, it has now been found that TZD rapidly and markedly increase IRS-2 gene expression. This effect was specific for PPARγ agonists and was not seen with PPARα agonists. It was rapidly induced (after 4 hrs) and maintained throughout the observation period of 48 hrs. It was also concentration-dependent and it was not inhibited by cycloheximide, suggesting a direct effect on the IRS-2 promoter. IRS-2 protein expression was also increased ~40 % after 48 hrs. No effects of TZD were seen on IRS-1, PKB/Akt or GLUT4 gene expression.

These data show the first direct link between TZD and a critical molecule in insulin's signaling cascade and indicate a novel mode of action of these compounds. This mechanism can now be utilised both for the screening of substances, in respect of their insulin sensitizing properties, and for developing pharmaceuticals for the treatment and prevention of diabetes.

Fig. 1A

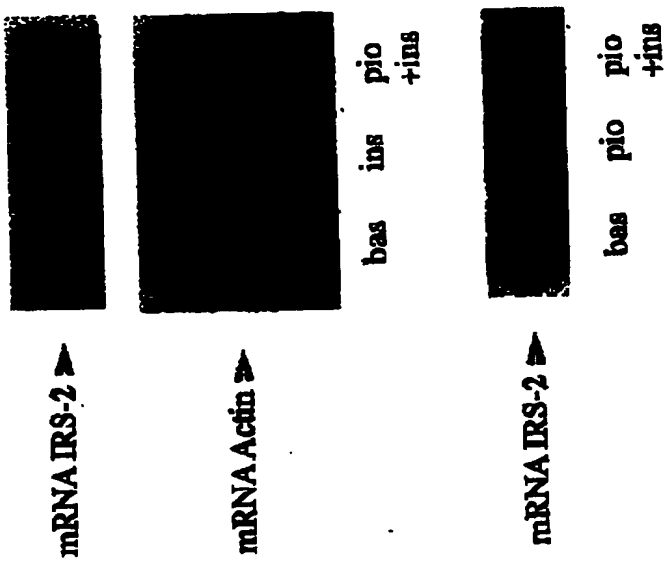
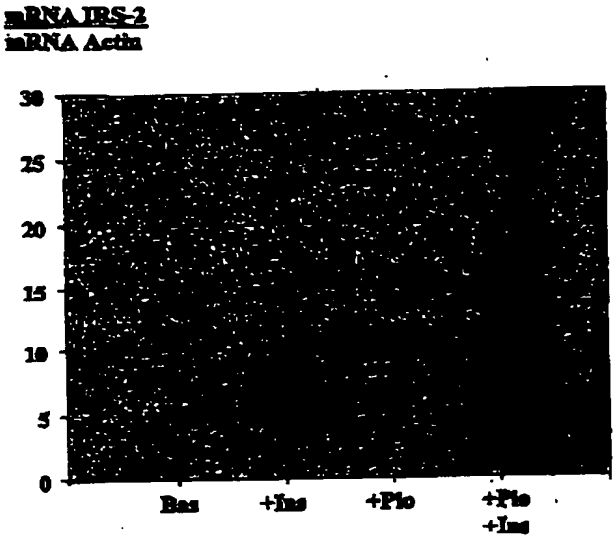
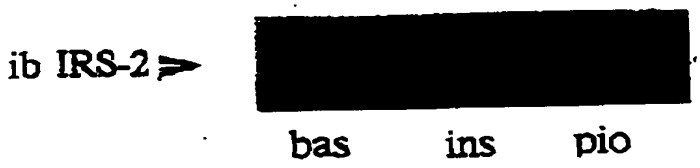


Fig.1B



IRS-2 ip



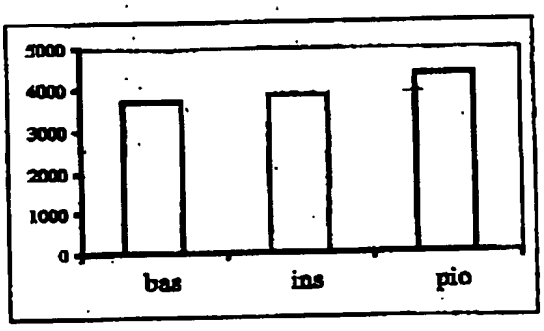


Fig.1C

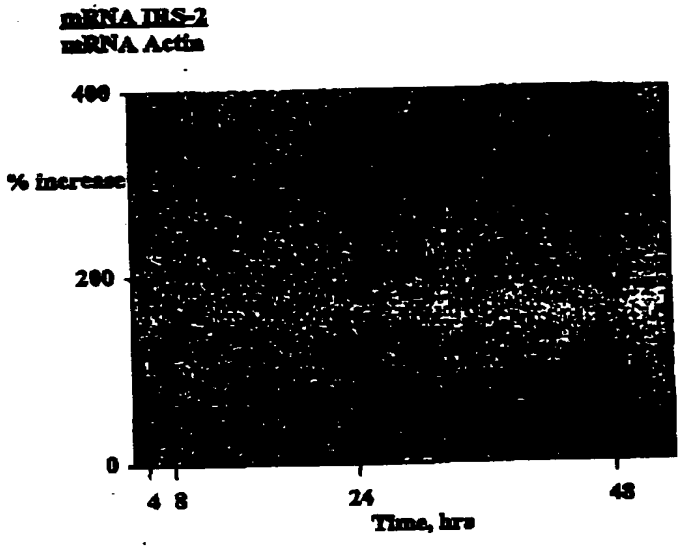
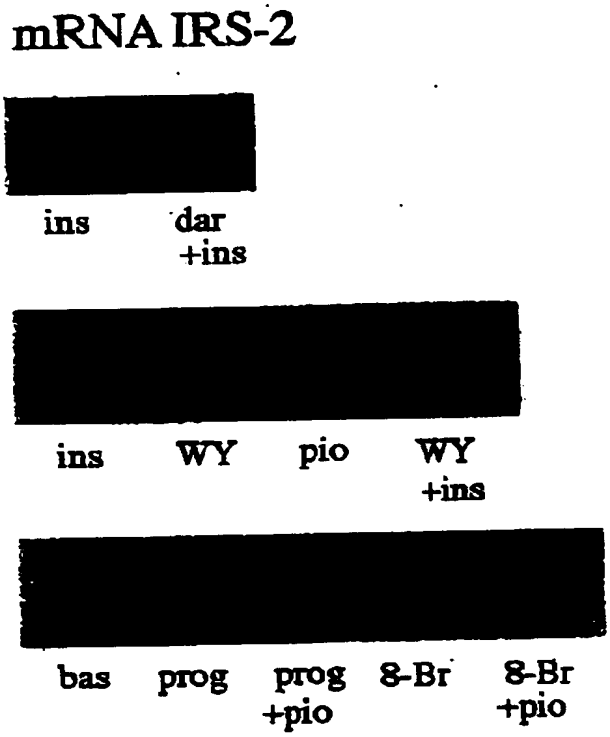


Fig. 2


mRNA IRS-2
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ins
WY
pio
WY +ins
bas
prog
prog +pio
8-Br
8-Br +pio

Fig. 3A

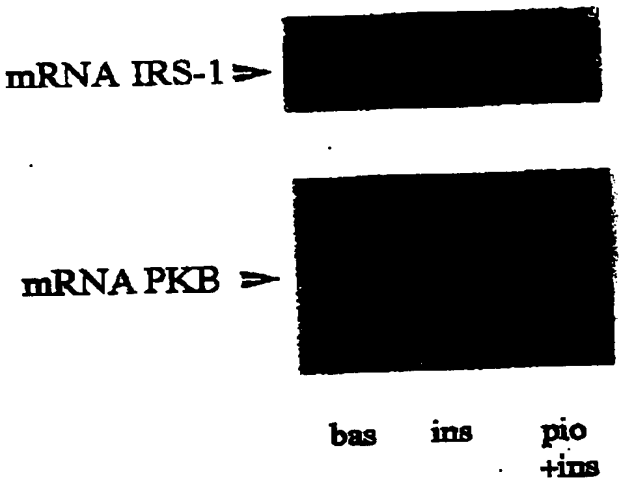
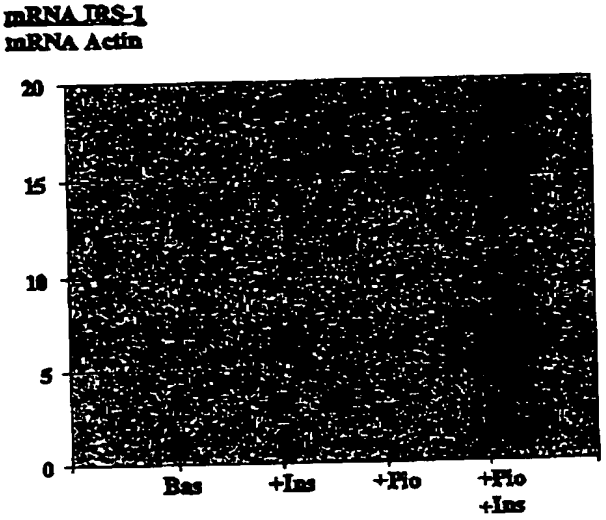


Fig.3B



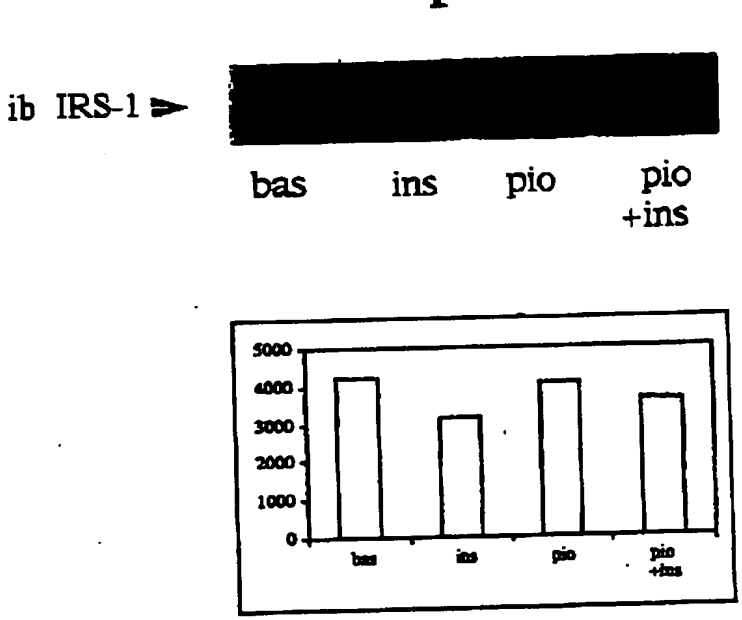


Fig. 4.